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Norovirus Genotypes Present in Oysters and in Effluent from a Wastewater Treatment Plant during the Seasonal Peak of Infections in Ireland in 2010

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We determined norovirus (NoV) concentrations in effluent from a wastewater treatment plant and in oysters during the peak period of laboratory-confirmed cases of NoV infection in Ireland in 2010 (January to March). Weekly samples of influent, secondary treated effluent, and oysters were analyzed using real-time quantitative reverse transcription-PCR for NoV genogroup I (GI) and genogroup II (GII). The mean concentration of NoV GII (5.87×10^4 genome copies 100 ml^{-1}) in influent wastewater was significantly higher than the mean concentration of NoV GI (1.40×10^4 genome copies 100 ml^{-1}). The highest concentration of NoV GII (2.20×10^5 genome copies 100 ml^{-1}) was detected in influent wastewater during week 6. Over the study period, a total of 931 laboratory-confirmed cases of NoV GII infection were recorded, with the peak ($n = 171$) occurring in week 7. In comparison, 16 cases of NoV GI-associated illness were reported during the study period. In addition, the NoV capsid N/S domain was molecularly characterized for selected samples. Multiple genotypes of NoV GI (GI.1, GI.4, GI.5, GI.6, and GI.7) and GII (GII.3, GII.4, GII.6, GII.7, GII.12, GII.13, and GII.17), as well as 4 putative recombinant strains, were detected in the environmental samples. The NoV GII.4 variant 2010 was detected in wastewater and oyster samples and was the dominant strain detected in NoV outbreaks at that time. This study demonstrates the diversity of NoV genotypes present in wastewater during a period of high rates of NoV infection in the community and highlights the potential for the environmental spread of multiple NoV genotypes.

Norovirus (NoV) is the leading cause of acute gastroenteritis outbreaks in Ireland (1) and is responsible for an estimated 90% of all epidemic nonbacterial outbreaks of gastroenteritis worldwide (2). NoV is highly infectious, with transmission occurring predominantly through person-to-person contact or by ingestion of fecally contaminated food or water (3). The majority of NoV outbreaks reported in Ireland occur in health care settings (4) and follow the pattern of winter seasonality demonstrated previously in the Northern Hemisphere (5).

NoV is a distinct genus of the family *Caliciviridae* that contains viruses with a positive-sense, polyadenylated RNA genome of approximately 7.5 kb. The genome of human NoV is organized into three open reading frames (ORFs). An NoV recombination hot spot has been identified close to the ORF1-ORF2 junction (6), and at least 22 NoV recombinants have been detected (7). The lack of a proofreading ability of the RNA-dependent RNA polymerase (RdRp) contributes to a high error rate of NoV genome transcription, resulting in even greater genetic diversity (8). Five NoV genogroups have been recognized (GI to GV) and may be subdivided into at least 29 genotypes (9). However, NoV GII genotype 4 (GII.4) accounts for the majority of acute gastroenteritis outbreaks characterized worldwide.

NoV is shed at high concentrations in the feces of infected patients (10^8 to 10^{12} genomic copies per g) and may be shed for up to 8 weeks after symptoms have subsided (10). NoV particles may be detected in feces of both symptomatic and asymptomatic individuals (10, 11) and thus are commonly present in wastewater treatment plant (WWTP) effluents throughout the year (12, 13). Shellfish harvest areas may be affected by the discharge of wastewater, and oysters can accumulate NoV from the surrounding waters through the process of filter feeding. Under European regulations (14), assessment of the sanitary quality of shellfish harvest areas relies on the use of the bacterial indicator organism *Escherichia coli*. However, *E. coli* has been shown to be an inadequate indicator of viral contamination (15), and numerous NoV outbreaks have been caused by shellfish compliant with the current regulations (16–18). Furthermore, a characteristic of shellfish-related outbreaks is the presence of multiple NoV GI and GII genotypes in the feces of infected patients (19, 20), whereas outbreaks in closed or semiclosed settings are generally associated with a single GII genotype (3), most commonly GII.4 (21).

In the present study, we investigated NoV concentrations in both influent and secondary treated effluent at a WWTP and in oysters placed adjacent to the WWTP discharge pipe over a 13-week period. In addition, the NoV concentrations/genogroups detected were subsequently compared with the number of laboratory-confirmed gastroenteritis cases caused by NoV GI and GII identified from outbreaks (community and food related) investigated during that time. Finally, we characterized the NoV genotypes detected during a 5-week period which had the highest NoV concentrations in municipal wastewater.

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MATERIALS AND METHODS

Sample population. A WWTP providing secondary wastewater treatment and serving a population equivalent of 91,600 was selected for this study. The final effluent was discharged to coastal waters through a 420-m-long outfall pipe submerged at a depth of 10 m. One-liter, 24-hour composite samples of influent ($n = 13$) and effluent ($n = 13$) were taken on a weekly basis from January to March (weeks 1 to 13) of 2010 and analyzed for NoV GI and NoV GII by real-time quantitative reverse transcription-PCR (RT-qPCR). Oysters (*Crassostrea gigas*) previously demonstrated to be free of NoV contamination were suspended in mesh bags 1 m below the water surface and attached to a buoy anchored to the outfall pipe. Oyster samples ($n = 13$), each consisting of a minimum of 10 animals, were collected 5 days after the wastewater samples, on a weekly basis (weeks 2 to 14). Oyster and wastewater samples were transported to the laboratory within 2 h under chilled conditions (<15°C). The greatest concentrations of NoV in the influent and effluent wastewater samples were detected between weeks 5 and 9 of 2010, and these samples were selected for further molecular characterization. Oyster samples corresponding to the selected wastewater samples (weeks 6 to 10) were also included in the sequencing analysis.

Stool samples ($n = 2,734$) received from symptomatic patients throughout Ireland between January and March 2010 were analyzed for the presence of NoV RNA. The majority of the samples were received from health care settings. Stool samples received from representative outbreaks ($n = 8$) that occurred between weeks 5 and 10 of 2010 were molecularly characterized. One NoV-positive stool sample was randomly selected per NoV outbreak.

Preparation of wastewater concentrate and shellfish proteinase K extract. A membrane filter adsorption-elution method was used for the concentration of wastewater samples as previously described (12). Briefly, 40 ml of each wastewater sample was passed through a cellulose prefilter (Millipore, Billerica, MA) placed directly on a negatively charged filter (Millipore) with a 0.45-μm pore size, using a vacuum pump system. Twenty-five milliliters of 0.14 M NaCl was passed through the filters, which were then placed in 4 ml of 50 mM glycine-NaOH buffer (pH 9.5) and mixed for 20 min at 500 rpm. The virus eluate was transferred to an Amicon Ultra-4 (50 kDa) (Millipore, Billerica, MA) centrifugal filter and centrifuged at 4,000 × *g* for 10 min. The retentate was resuspended in 550 μl of molecular biology-grade water, and the wastewater concentrate was stored at −20°C prior to RNA extraction.

Oyster samples were cleaned by rinsing under potable water and were opened using a flame-sterilized shucking knife. For each sample, the digestive tissues (DT) of 10 oysters were dissected and chopped, and 2 g was transferred to a 50-ml tube containing 2 ml of 100 μg ml^{-1} proteinase K solution (Sigma-Aldrich), followed by the addition of 50 μl of Mengo virus strain MC_0 as an internal positive control to control for extraction efficiency. This mixture was then incubated at 37°C with shaking for 60 min, followed by incubation at 60°C for 15 min. Following centrifugation at 3,000 × *g* for 5 min, the shellfish proteinase K extract (supernatant) was stored at −80°C prior to RNA extraction within 1 month.

RNA extraction procedure for shellfish and wastewater. Viral RNA was extracted from 500 μl of either shellfish proteinase K extract or wastewater concentrate by use of a NucliSENS miniMAG extraction platform and NucliSENS magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. An RNA extraction negative control (molecular biology-grade water) was included with each batch of samples extracted. Viral RNA was eluted into 100 μl of elution buffer and stored at −80°C. Additionally, each shellfish sample was extracted at three different dilutions of shellfish proteinase K extract (undiluted, 1:2, and 4:1), using phosphate-buffered saline (Oxoid, Basingstoke, United Kingdom), and then eluted in 30 μl of elution buffer prior to the nested RT-PCR.

NoV GI and GII quantification using one-step RT-qPCR. A previously described RT-qPCR assay was used to detect and quantify both NoV GI and GII in the influent, effluent, and oyster samples (12). For NoV GI analysis, previously described primers QNIF4 (22) and NV1LCR and probe NVGG1p (23) were used, and for NoV GII analysis, primers QNIF2 (24) and COG2R (25) and probe QNIFS (24) were used. For the internal positive control, primers Mengo209 and Mengo110 and probe Mengo147 were the same as those described by Pintó et al. (26).

pGEM-3Zf(+) plasmids carrying NoV GI and GII target sequences containing a restriction site (BamHI) to check for contamination (supplied by Francoise S. Le Guyader, Ifremer, Nantes, France) were used to enable quantification of NoV RNA, reported in copies per μl. A log dilution series of GI and GII DNA plasmids (range, 1×10^1 to 1×10^5 copies per μl) was included in duplicate in each RT-qPCR run, and the number of NoV RNA copies per μl was determined. The limit of detection (LOD) for NoV GI and GII was 20 genome copies g^{-1} of DT and 25 genome copies 100 ml^{-1} for shellfish and wastewater samples, respectively. All the samples were assessed for RT-PCR inhibition by using external control RNA (12). Samples with an amplification efficiency of <25% were not accepted, and in such cases, the sample RNA was reanalyzed at a 1:10 dilution. All the samples were also assessed for extraction efficiency, using a Mengo virus as an internal process control. Samples with a >1% extraction efficiency were accepted for inclusion in this study.

Molecular characterization and genotyping of NoV. A selection of influent and effluent wastewater, oyster, and NoV outbreak samples received between weeks 5 and 10 of 2010 were further characterized and sequenced as follows. Eight microliters of RNA was treated with 1 U of RNase-free DNase (Promega, United Kingdom) according to the manufacturer's protocol. RT was performed in a 30-μl reaction volume containing a 1 mM concentration of each deoxynucleoside triphosphate (dNTP), 10 mM dithiothreitol, 0.75 μg of random hexamers, 33 U RNase inhibitor, 300 U of SuperScript II reverse transcriptase (Invitrogen, United Kingdom), and 4.5 μl Superscript II buffer as described previously (27). RT was carried out at 42°C for 75 min, followed by an inactivation step at 99°C for 5 min. The first-round nested PCR mixture contained 5 μl cDNA and final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 20 μM (each) dNTPs, 2 μM (each) primers, 2.5 mM $MgCl_2$, and 2.5 U of AmpliTaq DNA polymerase (Applied Biosystems). The primers used for the NoV GI and NoV GII reactions were primers COG1F and G1-SKR and primers COG2F and G2-SKR, respectively (25, 28). The first-round PCR product (1 μl) was subsequently added to 49 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 20 μM (each) dNTPs, 0.4 μM (each) primers, 2.5 mM $MgCl_2$, and 2.5 U of AmpliTaq DNA polymerase. In the nested RT-PCR, the primers used were GISKF and GISKR for NoV GI and GIISKF and GIISKR for NoV GII (28). The PCR was performed under the following conditions: an initial denaturation at 95°C for 5 min; 40 amplification cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min; and a final extension of 72°C for 15 min. Amplified DNA fragments were purified using Chroma Spin columns (Unitech), and products were cloned into the pCR4-TOPO vector according to the protocol for a TOPO TA cloning kit (Invitrogen). Approximately 4 to 6 clones were randomly selected for DNA sequencing. PCR products were sequenced using a BigDye Terminator v 3.1 ready reaction kit (Applied Biosystems) per the manufacturer's recommendations and were analyzed on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Ten percent fecal suspensions were prepared in Star buffer (Roche) and were extracted (350 μl) by use of an MDX automated extractor (Qiagen), using the QIAamp One-For-All protocol per the manufacturer's instructions. Brome mosaic virus (BMV) (0.05 pg μl^{-1}) internal control RNA was added to the lysis buffer prior to extraction. A multiplex real-time RT-PCR was carried out using a Superscript III Platinum one-step quantitative RT-PCR kit (Invitrogen) per the manufacturer's instructions, with a 25-μl reaction volume. The primer and probe sequences for NoV were taken from the work of Rolfe et al. (29) and Kageyama et al. (25) and were used at concentrations of 400 nM and 80 nM for the primers and probes, respectively. BMV was coamplified by use of in-house primers and probes, at concentrations of 200 nM and 100 nM, respectively. Primer

TABLE 1 Mean concentrations of NoV GI and GII in influent and effluent wastewaters and impacted oysters between January and March 2010

NoV genogroup	Mean concn ± SD (range)		
	Influent[a]	Effluent[a]	Oysters[b]
GI	3.93 ± 0.44 (3.08–4.76)	3.01 ± 0.63 (1.73–4.06)	4.43 ± 0.45 (3.50–5.13)
GII	4.58 ± 0.38 (3.98–5.34)	3.44 ± 0.47 (2.34–3.99)	4.84 ± 0.25 (4.39–5.23)

[a] Concentrations are expressed as log genome copies 100 ml^{-1}.
[b] Concentrations are expressed as log genome copies g^{-1}.

and probe sequences for BMV were as follows: BMV F, 5′-CCT TTT TCA CTG CTT GTT TCG AGA A-3′; BMV R, 5′-TTT CCG ATA GGC ACA ATG AAC CT-3′; and BMV probe, 5′-NED-ACT GGC CCT GAA ATC-NFQ (MGB) (Applied Biosystems).

Phylogenetic analysis. The NoV sequences were aligned with reference strains retrieved from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) by using the ClustalW algorithm of MegAlign software (DNAStar, Inc., Madison, WI). The genotype nomenclature of the reference strains was adopted from the online Norovirus Genotyping Tool (http://www.rivm.nl/mpf/norovirus/typingtool; National Institute of Public Health and the Environment, Netherlands). The length of the NoV genome region used was 282 bp for NoV GI (positions 5354 to 5645 of Norwalk virus M87661) and 269 bp for NoV GII (positions 5085 to 5353 of Lordsdale virus X86557). NoV bovine strain Newbury GIII.2 was used as an outgroup strain in the phylogenetic analysis of NoV GI and GII, and the GII.3 strain Oberhausen AF53944 was used as an outgroup for GII.4 analysis.

A maximum likelihood phylogenetic tree was constructed using PAUP*, version 4.0 (30). Modeltest was used to select the most appropriate model of evolution, using the hierarchal likelihood ratio test (31). The maximum likelihood phylogenetic tree was based on the TrN+I+G model of substitution for NoV GI, the TrNef+I+G model for NoV GII, and the K80+G model for NoV GII.4 (31). The reliability of the generated tree was estimated by bootstrap analysis of 1,000 replicates of the sequence alignment, using the neighbor-joining method.

Recombinant analysis. Detection of potential NoV GI and GII recombinant sequences was carried out using the Recombination Detection Program, version 4.14 (RDP4) (32), comprising the following programs: RDP, GENECONV, BOOTSCAN, MaxChi, CHIMAERA, SISCAN, and 3SEQ. The default settings for each of the programs in RDP4 were used, except for BOOTSCAN and SISCAN, where a window size of 40 and a step size of 20 were used. The potential NoV recombinants were considered putative recombinant sequences if they were detected by at least 3 different programs.

Statistical analysis. The Anderson-Darling test for normal distribution was applied to each data set, and the NoV concentrations in wastewaters and oysters were logarithmically (base 10) transformed to achieve a normal distribution. Minitab statistical software, version 16 (Minitab Inc., State College, PA), was used for the data analysis. The reductions during the wastewater treatment process were calculated on a weekly basis by using the following equation: log reduction = $\log(N_{inf}/N_{eff})$, where N_{inf} is the concentration of NoV detected in influent wastewater and N_{eff} is the concentration of NoV detected in effluent wastewater.

Nucleotide sequence accession numbers. The GenBank accession numbers for all sequences analyzed during this study are as follows: JQ362499 to JQ362594 and JQ280400 to JQ280407.

RESULTS

NoV concentrations in influent, effluent, and oysters between January and March 2010. NoV GI and GII were detected in influent, effluent, and oyster samples on all sampling occasions throughout the sampling period. The mean NoV GI and GII concentrations detected in influent wastewaters were 3.93 and 4.58 log genome copies 100 ml^{-1}, respectively (Table 1). The mean concentration of NoV GII was 0.66 log genome copies 100 ml^{-1} higher than the mean concentration of NoV GI in influent wastewater, and this difference was significant (paired *t* test; $P < 0.001$). The mean NoV GI and GII concentrations detected in effluent wastewater were 3.01 and 3.44 log genome copies 100 ml^{-1}, respectively, and were not statistically different (paired *t* test; $P > 0.05$). The mean concentrations of NoV GI and GII detected in oyster samples were 4.43 and 4.84 log genome copies g^{-1}, respectively, and this difference was statistically significant (paired *t* test; $P = 0.005$). The mean NoV GI and GII reductions during the treatment process were 0.92 and 1.14 log genome copies 100 ml^{-1}, respectively.

Laboratory-confirmed cases of NoV gastroenteritis. During the study period, NoV GI and GII RNAs were detected in 16 and 931 stool samples, respectively. A peak in laboratory-confirmed cases occurred in week 7 of 2010, with 171 patient samples testing positive for NoV GII (Fig. 1). The largest number of laboratory-confirmed cases of NoV GI infection ($n = 4$) were detected in week 9 of 2010. Overall NoV GII cases were confirmed approximately 58.2 times more frequently than NoV GI cases ($P < 0.001$). The highest NoV GII concentration was detected in influent wastewater in week 6 of 2010 (2.20×10^5 copies 100 ml^{-1}), which preceded the peak of laboratory-confirmed cases involving NoV GII, in week 7. In the influent, the highest concentration of NoV GI (5.59×10^4 copies 100 ml^{-1}) was detected in week 7.

Multiple NoV genotypes detected in wastewater, oyster, and stool samples. Following phylogenetic characterization of samples from weeks 5 to 10, NoV GI sequences ($n = 73$) were ascribed to five different genotypes, namely, GI.1, GI.4, GI.5, GI.6, and GI.7 (Fig. 2), and NoV GII sequences ($n = 74$) were ascribed to seven genotypes, namely, GII.3, GII.4, GII.6, GII.7, GII.12, GII.13, and GII.17 (Fig. 2). Among NoV GII.4 genotypes, three variants were identified: 2006b, 2008, and 2010. NoV sequences with 100% identity detected in the different samples were designated groups A to E for NoV GI and groups F and G for NoV GII (Fig. 2).

NoV outbreaks ($n = 8$) that occurred between weeks 5 and 10 of 2010 (Fig. 1) were genetically characterized and incorporated into the phylogenetic analysis (Fig. 2). Outbreaks were associated with NoV GII.4 variant 2010 ($n = 5$), NoV GII.4 variant 2006b ($n = 1$), NoV GII.13 ($n = 1$), and NoV GI.6 ($n = 1$). A NoV GI.6 sequence (10IRL06528) (Fig. 2) associated with an outbreak that occurred during week 9 had 100% sequence identity with NoV sequences detected in effluent and oyster samples collected during week 8 (group D) (Fig. 2).

Norovirus genotype patterns in wastewater and oysters. In five samples of influent wastewater ($n = 25$ sequences), the most frequently detected NoV GI genotype was GI.7 (15/25 sequences [60.0%]), followed by GI.5 (5/25 sequences [20.0%]), GI.4 (4/25 sequences [16.0%]), and GI.6 (1/25 sequences [4.0%]) (Fig. 3). In five samples of effluent wastewater ($n = 25$ sequences), NoV GI.4 was the most prevalent NoV GI

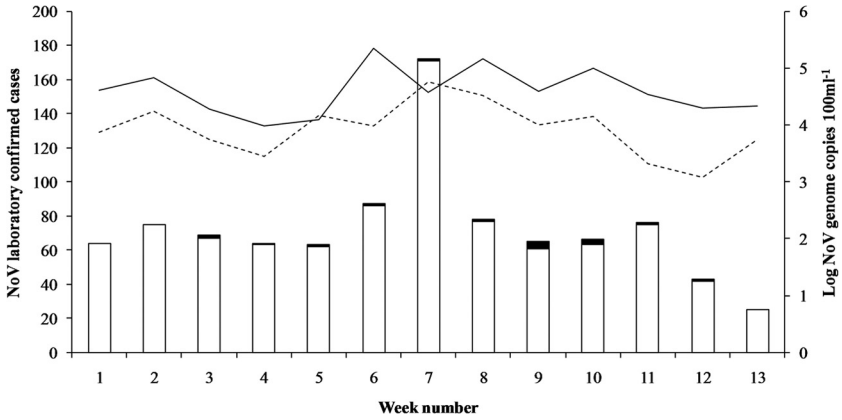

FIG 1 Laboratory-confirmed NoV cases and NoV concentrations in influent wastewater. The numbers of gastroenteritis cases attributed to NoV GI (black bars) and NoV GII (white bars) are shown, along with concentrations of NoV GI (dashed line) and NoV GII (solid line) detected in influent wastewaters between January and March 2010 (weeks 1 to 13). One NoV-positive stool sample per NoV outbreak was randomly selected for further sequencing analysis. Eight NoV outbreaks were genetically characterized as follows: in week 5, one outbreak sequence, 10IRL03196, was identified as GII.4 2010; in week 6, two outbreak sequences, 10IRL02653 and 10IRL02654, were identified as GII.4 2010; in week 7, outbreak sequences 10IRL04566 and 10IRL07388 were identified as GII.4 2010, 10IRL03903 was identified as GII.13, and 10IRL05499 was identified as GII.4 2008; and in week 9, outbreak sequence 10IRL06528 was identified as GI.6.

genotype, detected in 44.0% of the sequences (11/25 sequences), followed by GI.7 (10/25 sequences [40.0%]) and GI.6 (4/25 sequences [16.0%]). In oyster samples, NoV GI.4 was also the predominant NoV GI genotype (13/23 sequences [56.5]), and the other genotypes identified were GI.6 (5/23 sequences [21.7%]), GI.7 (3/23 sequences [13.0%]), GI.5 (1/23 sequences [4.3%]), and GI.1 (1/23 sequences [4.3%]) (Fig. 3).

NoV GII.4 was the dominant NoV GII genotype detected (8/24 sequences [33.3%]) in influent wastewater, followed by GII.12 (6/24 sequences [25.0%]), GII.17 (4/24 sequences [16.7%]), GII.6 (3/24 sequences [12.5%]), GII.7 (2/24 sequences [8.3%]), and GII.13 (1/24 sequences [4.2%]). In the effluent wastewater, the dominant genotype of NoV sequences was GII.4 (13/24 sequences [54.2%]), also followed by GII.6 (6/24 sequences [25.0%]), GII.12 (3/24 sequences [12.5%]), and GII.13 (2/24 sequences [8.3%]). Genotypes GII.7 and GII.17 were not detected in the effluent, despite being detected in the influent wastewater. In oysters, two genotypes, GII.4 (10 sequences [38.5%]) and GII.12 (10 sequences [38.5%]), were detected at the same frequency, followed by GII.3 (4 sequences [15.4%]) and GII.6 (2 sequences [7.7%]). Two genotypes, NoV GI.1 and GII.3, were detected in oysters, but these were not detected in wastewater samples (Fig. 3B).

Recombinant analysis. Four potential NoV recombinant strains were identified following analysis by RDP4. A sequence was considered to be a putative recombinant if a recombination event was detected by at least 3 recombinant detection methods. The parent genotype and putative recombinants detected were as follows: GII.12/GII.4 2006b (recombinant EFFII03035), GII.4 2010/GII.13 (recombinant INFII03039), and GII.4 2010/GII.3 (recombinant OYSII09021) for NoV GII and GI.4/GI.6 for NoV GI (Fig. 4). Potential parental sequences and possible recombination breakpoints are listed in Table 2.

DISCUSSION

In 2010, 1,789 NoV illness notifications were reported in Ireland. This was the largest number of NoV notifications compared to the numbers in previous years. A particularly large number of NoV notifications (1,309) was recorded between January and March (1). In the first 3 months of 2010, increased numbers of NoV infections were also reported in other parts of Europe, including Scotland (33), England and Wales (34), and Belgium (35). Higher-than-average NoV activity in epidemic years has been associated with cold, dry weather, lower population immunity, and the emergence of new NoV GII.4 variants (36). The winter of 2009–2010 (December to February) was the coldest winter since the winter of 1962–1963 in Ireland, but it was drier and sunnier than usual, as reported by Met Éireann (http://www.met.ie/).

The national peak of NoV GII infections, as judged by laboratory-confirmed cases, occurred approximately 1 week after the highest concentration of NoV GII was detected in influent wastewater. However, given that NoV shedding can commence before the onset of clinical symptoms (10) and that there is a slight time lag between infection and laboratory confirmation, it is likely that the peak concentration of NoV GII in wastewater occurred concurrently with the peak number of infections in the community. Detection of elevated NoV GII concentrations in wastewater prior to higher-than-normal reports of NoV gastroenteritis has been described previously (22). Few NoV GI-associated laboratory-confirmed cases were reported compared to those involving NoV GII, and 7 of a total of 16 cases occurred between weeks 9 and 10. However, given the small number of overall NoV GI-associated cases, it is not possible to determine whether this represented a peak of infections.

Concentrations of NoV GII were significantly greater than NoV GI concentrations detected in influent wastewater, indicating either a greater frequency of NoV GII-associated infections in the community or greater virus shedding by NoV GII-infected patients than by NoV GI-infected patients. During the wastewater treatment process, both NoV GI and GII underwent comparable reductions over the study period, and the achieved reductions (approximately 1 log) are consistent with reductions in previously reported studies (13, 37). Larger numbers of laboratory-confirmed cases of NoV GII infection were detected in the community. It appears that the greater concentrations of NoV GII in the influent wastewater were a result of NoV GII cases in the community. Nevertheless, despite the small number of laboratory-con-

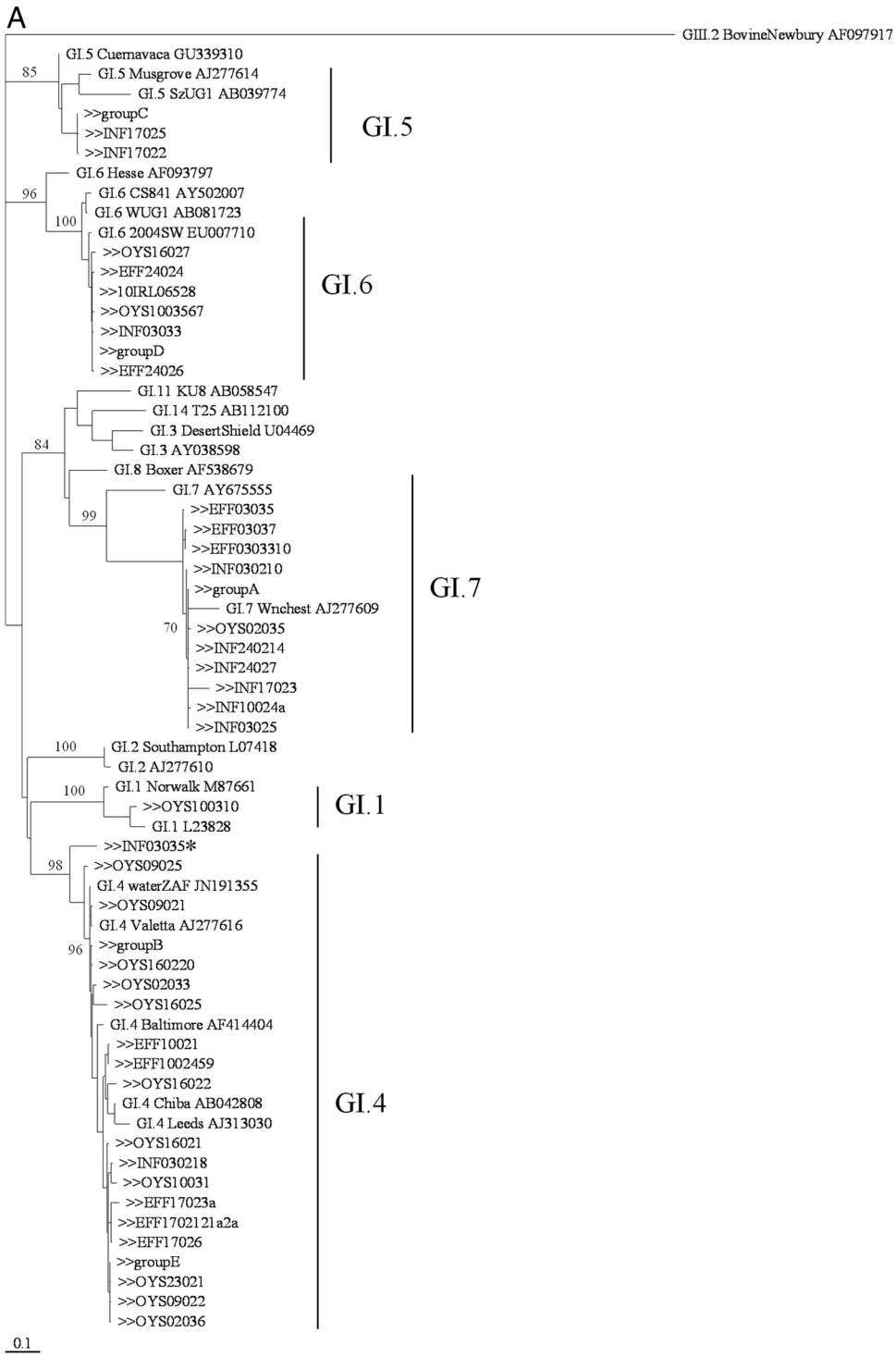

FIG 2 Molecular characterization of NoV GI (A) and GII (B) sequences detected in wastewater, oysters, and gastroenteritis outbreaks in Ireland between January and March 2010. Phylogenetic trees were based on the partial capsid region of the NoV GI and GII genomes (282 and 269 bp, respectively). For NoV GII.4 sequences, a separate phylogenetic tree was built. All phylogenetic trees include bootstrap scores for branches, shown as percentages for 1,000 replicates (bootstrap values of <70% are not shown). NoV sequences were named with the code "INF" (influent), "EFF" (effluent), or "OYS" (oysters) followed by *xx* (sampling day) and *xx* (sampling month), followed by consecutive numbers used for laboratory differentiation. Identical sequences of NoV GI detected in different samples were designated group A (influent sampled on weeks 5, 6, 7, and 8, effluent sampled on weeks 5 and 9, and oysters sampled on week 9), group B (oysters from weeks 6 and 8), group C (influent from weeks 7 and 9 and oysters from week 8), group D (effluent from week 8 and oysters from week 8), or group E (influent from weeks 5 and 9). The NoV GI sequence detected in the NoV outbreak was labeled 10IRL06528. Identical NoV GII sequences detected in different samples were designated group F (influent sampled on weeks 5 and 7 and effluent sampled on week 8), group G (effluent from weeks 6 and 7), or group H (oysters from weeks 8 and 9). NoV GII sequences detected in the NoV outbreak were labeled 10IRL02653, 10IRL02654, 10IRL03196, 10IRL03903, 10IRL04566, 10IRL05499, and 10IRL07388. *, putative recombinant.

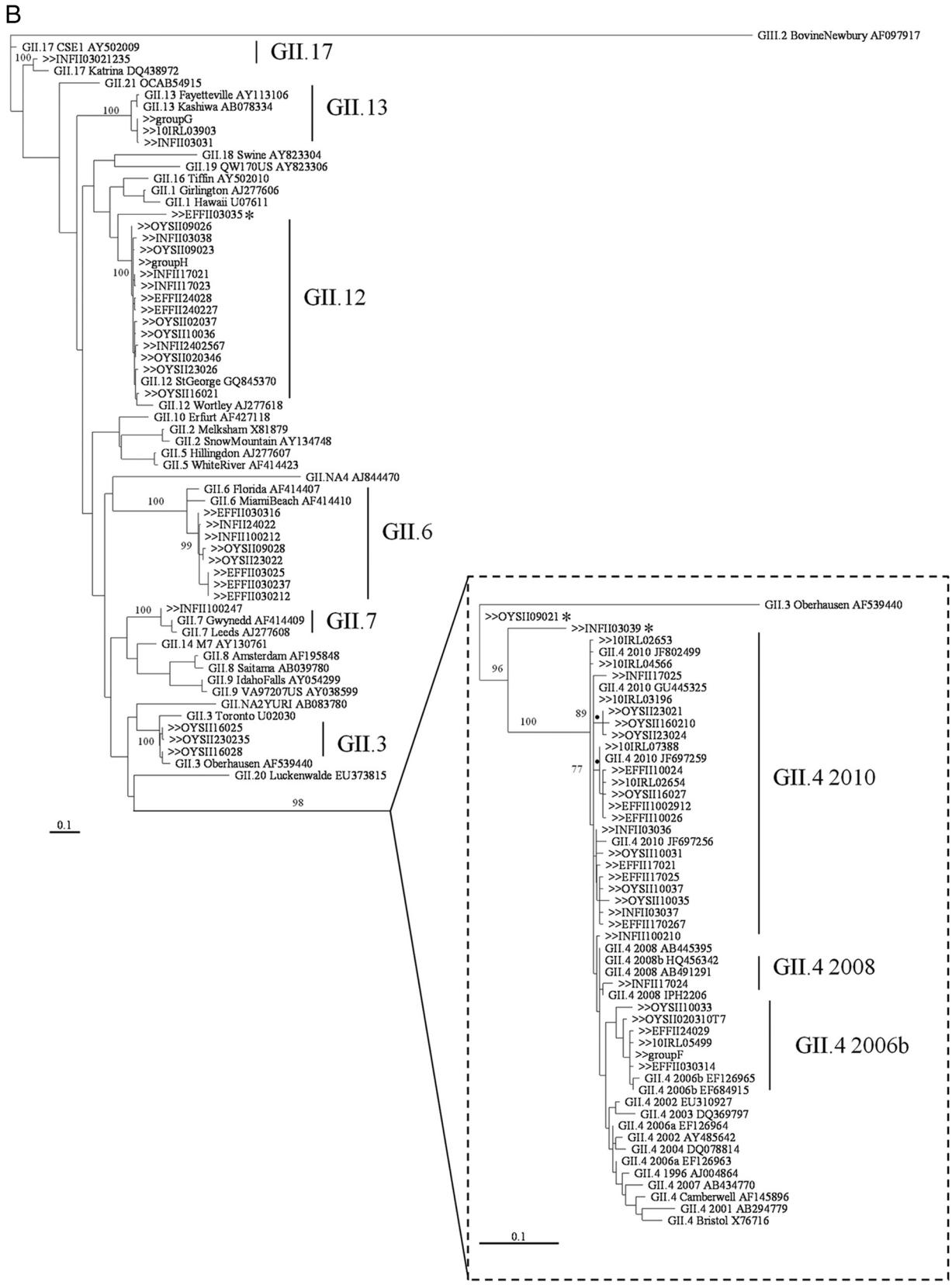

FIG 2 continued

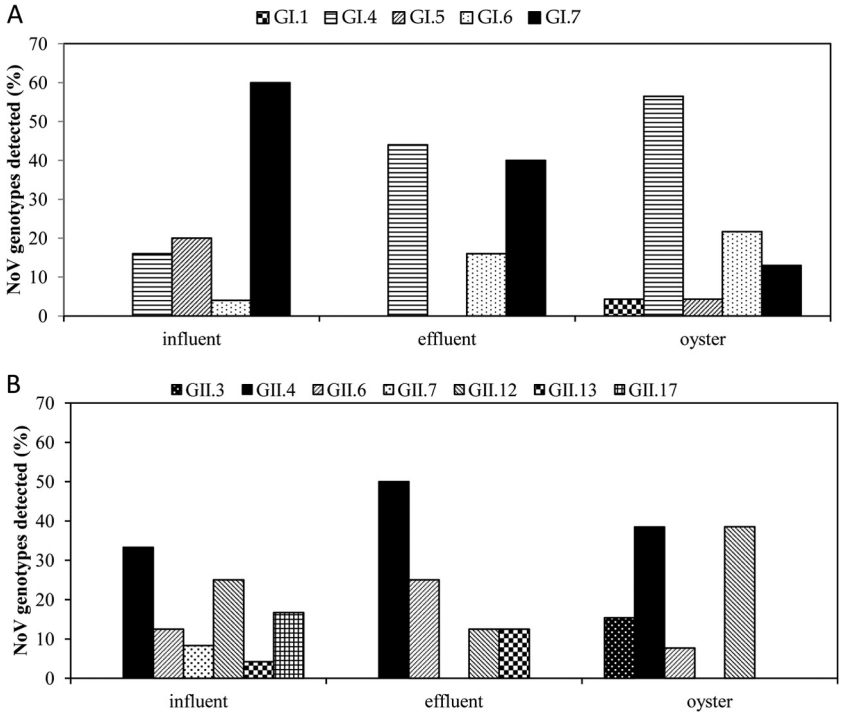

FIG 3 NoV genotype profiles detected in wastewater and oyster samples. NoV GI genotypes (A) and NoV GII genotypes (B) are shown as percentages of all genotypes detected in either the influent or effluent wastewater or oyster samples.

firmed cases of NoV GI infection during the study period, relatively high concentrations of NoV GI were detected in influent wastewater, and concentrations peaked in week 7. This suggests that during the study period, an increased number of community infections associated with NoV GI occurred. Unless a significant number of NoV GI-associated infections are asymptomatic, it appears that NoV GI-associated infections were underreported in Ireland during this study period. The majority of stool samples analyzed in this study were obtained from outbreaks that occurred in health care settings. Given the previously described prevalence of NoV GII infections in such settings (3, 4), the large number of laboratory-confirmed cases involving NoV GII was expected. It is possible that NoV GI infections are underreported to public health authorities more often than NoV GII infections due to

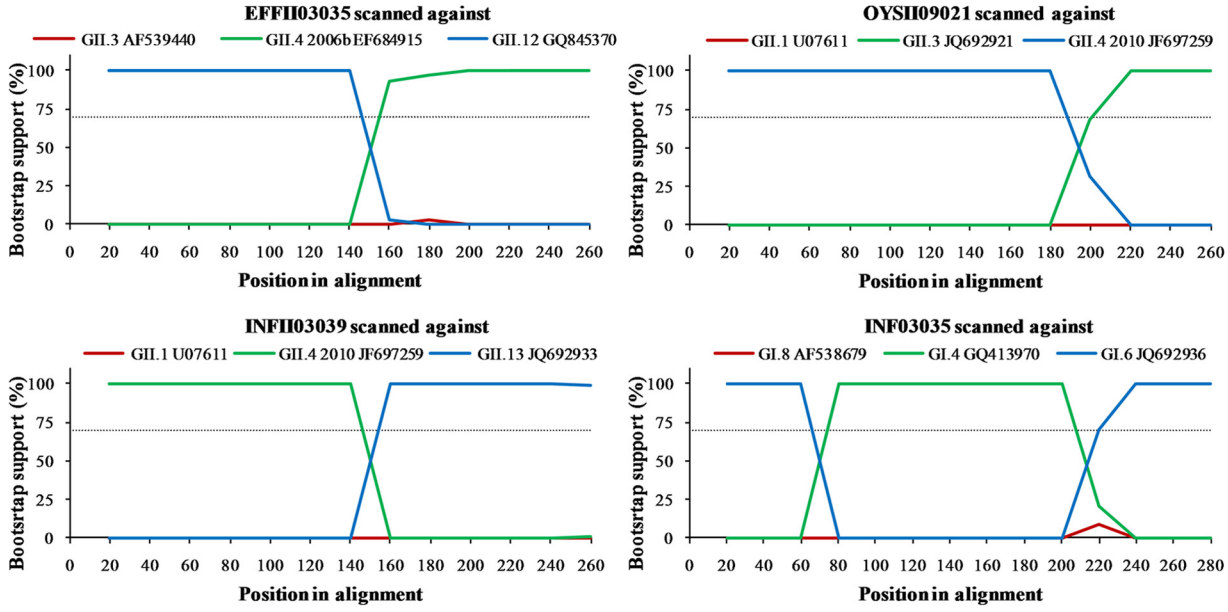

FIG 4 BOOTSCAN evidence of recombination events. BOOTSCAN analysis was performed based on pairwise distance, with a window size of 40, a step size of 20, and 100 bootstrap replicates.

TABLE 2 Recombination events with possible recombination breakpoints and *P* values for the different recombination detection methods

NoV	Recombinant sequence	Breakpoint[a]		*P* value for detection method[b]					
		Beginning	End	GENECONV	BOOTSCAN	MaxChi	CHIMAERA	SISSCAN	3SEQ
GII	EFFII03035	Unidentified	152 (5236)	1.10×10^{-4}	4.19×10^{-7}	7.15×10^{-7}	7.15×10^{-7}	NS	6.55×10^{-17}
GII	INFII03039	Unidentified	147 (5231)	4.29×10^{-3}	9.29×10^{-6}	2.72×10^{-6}	2.68×10^{-6}	NS	2.19×10^{-13}
GII	OYSII09021	Unidentified	201 (5285)	NS	1.01×10^{-4}	5.02×10^{-6}	4.95×10^{-6}	NS	1.05×10^{-13}
GI	INF03035	75 (5428*)	219 (5582*)	NS	1.17×10^{-3}	4.87×10^{-6}	1.01×10^{-5}	2.11×10^{-12}	2.60×10^{-12}

[a] Breakpoint position in alignment (breakpoint position relative to strain X86557 or M87661 [strain M87661 where marked with an asterisk]).
[b] NS, not significant.

sampling bias in health care settings, their more-varied transmission mode (3, 38), or the severity of illness (39).

NoV outbreaks were genetically characterized, and three different genotypes were detected: GII.4 (variants 2010 and 2006b), GII.13, and GI.6. All of these NoV sequences were highly similar to NoV sequences found in the influent wastewater, effluent wastewater, and oysters. The newly emerged GII.4 2010 variant (35, 40) was the most frequently detected variant of GII.4 in the outbreaks as well as in the environmental samples. The GII.4 2010 variant was first identified in France in February 2009 (40), and it became the dominant GII.4 variant in Europe during the winter of 2009–2010 (33, 35, 41). In the United States, GII.4 2010 replaced GII.4 2006b as the predominant variant in the winter of 2009–2010 (42, 43). GII.4 2010 gained a global distribution in 2009 and 2010, being detected in Taiwan, China, Australia, Japan, India, Cameroon, and the United States (BLAST search). It is clear that a similar GII.4 variant replacement took place in Ireland, as supported by the high rates of detection in wastewater and of laboratory-confirmed cases of NoV infections reported in this study.

Interestingly, all of the NoV GII.12 sequences detected in the environmental samples were highly similar to the recombinant strain GII.g (polymerase)/GII.12 (capsid) St. George (GenBank accession no. GQ845370) (44), which was reported in a large number of non-GII.4 NoV outbreaks in the United States (45, 46) and Europe (41) in the winter of 2009–2010. However, this strain was not identified in any of the outbreak samples analyzed in this study. It is possible that some of the NoV genotypes frequently identified in the influent, the effluent, and impacted oysters, such as NoV GII.12, were responsible for infections in the community at the beginning of 2010 but were not detected due to limited sequencing carried out on the outbreak samples.

The NoV genotype profiles detected in the influent, effluent, and oyster samples varied. NoV GI.7 and NoV GI.4 were the most frequently detected NoV GI genotypes from environmental samples during this study. This is consistent with the findings of the Foodborne Viruses in Europe (FBVE) network, which reported these genotypes as the most frequent causes of NoV GI outbreaks throughout 2010 (41). In our study, NoV GI.7 was detected predominantly in the influent, less frequently in effluent wastewater, and only sporadically in oyster samples. In contrast, NoV GI.4 was the dominant genotype detected in effluent wastewater and oysters. This is consistent with a previous study by this laboratory in which NoV GI.4 was the predominant genotype detected in oysters originating from a commercial harvest area (47). In this study, the NoV GI.1 and GII.3 genotypes were absent in wastewater but were detected in oyster samples, and this could possibly be linked with their preferential accumulation in oyster tissues, which has been reported previously (48).

Recent studies on virus-like particles (VLPs) have shown that the overall virion structures of GI.1, GII.4, GII.10, GII.12, and GV.1 VLPs are similar; however, differences in flexibility between their P and S capsid domains can influence binding of VLPs to monoclonal antibodies (49, 50). It has also been shown that NoV GI.1 particles can bind specifically to a histo-blood group antigen (HBGA) A-like ligand present in the oyster digestive tract, and this binding facilitates accumulation within oysters (48). Therefore, it is reasonable to suggest that some NoV genotypes may accumulate in oysters more efficiently than others because of their binding properties, which explains why some NoVs have been detected more commonly in shellfish-related outbreaks (51).

We identified four putative NoV recombinants: GI.4/GI.6 and GII.4 2010/GII.13 in the influent, GII.12/GII.4 2006b in the effluent, and GII.4 2010/GII.3 in the oysters. Although a short fragment (291 bp for GI and 302 bp for GII) of the NoV genome was used to identify recombinants, the detection of each recombinant was supported by at least three detection methods available in RDP4. The GII.4/GII.3 recombinant has been described previously by Vidal et al. (52); however, to our knowledge, the three other recombinants detected here have not been identified before. Most of the parental sequences identified, such as GI.6, GII.4 2010, GII.4 2006b, and GII.13, were detected in the clinical samples during this study. As the putative recombinants were detected in the environmental samples only, it is not possible to comment on their virulence. It has been shown experimentally (53) that a novel murine NoV recombinant can exhibit different biological properties from those of its parental viruses. As oysters can accumulate different NoV genotypes (including recombinants), the potential exists for the reintroduction of these new or possibly more virulent NoV strains to the human population through contamination of oysters and their subsequent consumption.

In summary, we suggest that during this study, allowing for the delay in laboratory confirmation, there were concurrent peaks in NoV GII concentrations in wastewater and NoV infections in the community in Ireland in February 2010. We demonstrated that a diverse range of NoV GI genotypes were present in environmental samples (wastewater and oysters), despite the fact that the overwhelming majority of laboratory-confirmed cases were attributed to NoV GII. Therefore, we suggest that the number of NoV GI-associated infections may be underreported. Finally, different genotype profiles were observed in influent wastewater and oysters, possibly indicating differing survival characteristics through the treatment process for different genotypes or preferential accumulation of some genotypes in oysters.

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